

Antimicrobial activity of methanolic *Nigella sativa* seeds and *Saussurea lappa* roots extracts on multidrug resistant Gram negative bacteria

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ABSTRACT

Different solvent extracts of *Nigella sativa* seeds and *Saussurea lappa* roots were investigated for their antimicrobial activity against some Gram negative bacteria isolated from clinical samples from neonatal intensive care unit (NICU) from King Khalid Hospital, Tabuk, Kingdom of Saudi Arabia. Ten clinical isolates, *Pseudomonas aeruginosa* (n=5), *Serratia marcescens* (n=2), *Proteus mirabilis* (n=2) and *Sphingomonas paucimobilis* (n=1). These isolates were multidrug resistant. The methanolic extract of *S. lappa* showed high antimicrobial activity rather than that of *Nigella sativa* seeds especially against *Sphingomonas sp.* and *Serratia sp.*. Both methanolic extracts of *S. lappa* roots and *Nigella sativa* seeds showed antibacterial activity against *Pseudomonas sp.* and *Proteus sp.* with varying extents. The chemical composition of the investigated two plant extracts was examined. It contained phenols, tannins and flavenoids. The emergence of antibiotic resistance and the high risk of the abuse of antibiotics, highlight the need of using alternative agents that have low risk and costless.

Key words: Antibacterial activity, *Nigella sativa*, *Saussurea lappa*, methanolic extract, Gram negative bacteria.

INTRODUCTION

Nigella sativa known as black seed (Benlafya *et al.*, 2014) is an herbaceous plant, belonging to the family Ranunculaceae (Chopra *et al.*, 1956). It was used for treatment of various ailments in different countries (Mammad *et al.*, 2017) such as antimutagenic, anti-inflammatory effects (Khadera *et al.*, 2010). Also, it has insulinotropic, hypoglycemic, anticancer, antibacterial properties (Feroz and Uddin, 2016). It is also used in folk medicine for a number of diseases such as eczema, asthma, gastrointestinal disturbances, inflammation, cough, hypertension, fever and diabetes (Ali and Blunden, 2003).

Large numbers of active ingredients have been reported in *Nigella sativa* seeds including nigellicine, carvacrol, dithymoquinone, a-hederin, thymohydroquinone, thymoquinone and thymol (Ali and Blunden, 2003). Thymoquinone has anti-inflammatory and analgesic actions (Abdel-Fattah *et al.*, 2000) and Thymol has antibacterial activity (Salman *et al.*, 2016).

Saussurea lappa is an endangered medicinal herb. It is indigenous to Himalayas, Pakistan, China and Kashmir Valley in India. It belongs to family Compositae (Asteraceae) (Choudhary, 2015). It is known by common names Kust (Arab and Persian), Kuth or Postkhai

(Kashmiri), Kut or Kur (Hindi and Bengali) (Madhuri *et al.*, 2012), Minal (Urdu), Costus (English) (Khalid *et al.*, 2011).

The roots of *Saussurea lappa* has many pharmacological important such as antibacterial, antifungal activity (Khalid *et al.*, 2011), antitumor, immunostimulant (Hamilton, 2004), antidiabetic, anthelmintic (Upadhyay *et al.*, 1996), anti-inflammatory, antiulcer and antihepatotoxic (Sutar *et al.*, 2011).

Many studies on *Saussurea lappa* roots had been done and many chemical compounds were isolated. Terpenes is the main active constituent and it is also contains anthraquinones, flavonoids (Zahara *et al.*, 2014), phenolic compounds (Prabakaran *et al.*, 2011), reducing sugars, tannins and alkaloids. Different research studies had emphasized the antibacterial activity of methanolic extract of *Saussurea lappa* against Gram negative bacterial strains (Thara and Zuhra, 2012).

The aim of this study is to evaluate the effect of methanolic extracts of *Nigella sativa* seeds and *Saussurea lappa* roots on some multidrug resistant (MDR) Gram negative bacteria isolated from clinical samples from neonatal intensive care unit from King Khalid Hospital, Tabuk, Kingdom of Saudi Arabia.

MATERIALS AND METHODS

Bacterial strains:

Bacterial strains:

Bacterial isolates were recovered from clinical samples from neonatal intensive care unit (NICU) from King Khalid Hospital, Tabuk, Kingdom of Saudi Arabia. The isolates were cultured on MacConkey agar and identified by API-20E test strips (Biomeriux, France) and were further confirmed using VITEK-2 AST-N 209 cards (Biomeriux, France).

Plant Collection:

Seeds of *Nigella sativa* and *Saussurea lappa* roots were purchased from local herbal shops.

Preparation of the plant extract:

N. sativa seeds and *S. lappa* roots were washed and dried at 40°C, then grinded to fine powder by using a mortar and stored in opaque screw tight jars until use as recommended by Ogundiya *et al.* (2006). Different organic solvents in increasing polarity order (petroleum ether 60-80, 80-100, ethyl acetate, methanol) were used according to Jeyaseelan *et al.* (2012).

50g of each plant powder was soaked in 150ml of the solvent, shaken at 100 rpm in 60°C for 24h, and then filtered. The residue was further extracted two times by using fresh solvent at the same conditions. All the filtrates were pooled and dried in freeze dryer to minimum volume. The yield of the each extraction was measured separately and the extracts were stored at 4°C until used (Doughari and Okafor, 2007).

Antibacterial activity test:

Antimicrobial activity of the methanolic extract of *N. sativa* seeds and *S. lappa* roots against the MDR Gram negative bacteria had been tested by well diffusion method. Bacterial cultures of 24h were prepared in saline solution with density adjusted to approximately 10^6 CFU/ml. Mueller Hinton agar medium was prepared and inoculated with 150µl of each test organism then spread over the agar plates using a sterile glass spreader.

Different concentrations (1.25, 2.5, 5.0, 10.0 and 20.0mg/ml) of the methanolic extract were prepared, 100 µl of each extract

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was added separately to the wells in the inoculated Petri dishes, distilled water was

used as a negative control (Aneja *et al.*, 2011). The experiments were carried out in triplicates and the average values of the diameter of inhibition zones were evaluated (Matthew *et al.*, 2013).

Chemical analysis:

The analysis of phytochemical content of each plant was carried out according to the methods of detection of flavonoids, saponins, alkaloids (Sofowora, 1993), and tannins according to Trease and Evans (1978). Tannins were detected by the addition of 1 ml of the sample taken in a test tube and then 1 ml of 0.008 M Potassium ferricyanide was added. 1 ml of 0.02 M Ferric chloride containing 0.1 N HCl was added and observed for blue-black coloration.

Test for saponins was carried out by mixing with crude extracts 5 ml of distilled water in a test tube and shaken vigorously. Some drops of olive oil were added. The formation of stable foam was taken as an indication for the presence of saponins. To test for flavonoids, 5 ml of dilute ammonia solution were added to a portion of the crude extract followed by addition of concentrated H₂SO₄. A yellow coloration observed in each extract indicated the presence of flavonoids. The yellow coloration disappeared on standing. The test for alkaloids was done by mixing the crude extracts with 2 ml of Wagner's reagent. Reddish brown colored precipitate indicated the presence of alkaloids. To test for sterols, Salkowski reaction was used, 0.5mg of residue of each extract was taken in 2ml of chloroform and 2ml of concentrated sulphuric acid was added from the side of the test tube. The test tube was shaken for

few minutes. The development of red color in the chloroform layer indicated the presence of sterols (Soni and Sosa, 2013). Tests for phenols and Ferric chloride were carried out, 1 ml of each extract was taken in water and warmed then 2 ml of ferric Chloride (FeCl₃) was added. The formation of green and blue colour solution indicates the presence of phenolic compounds. (Soni and Sosa, 2013).

RESULTS AND DISCUSSION

Bacterial isolates:

10 bacterial isolates were used in this study, they represent 4 different species of Gram negative bacteria and the isolates were recovered from different clinical samples as shown in Table (1). *Pseudomonas aeruginosa* (5 isolates), *Serratia marcescens* (2 isolates) and *Proteus mirabilis* (2 isolates) and *Sphingomonas paucimobilis* (one isolate). All isolates were considered MDR as they were resistant to more than 3 groups of antibiotics.

The MIC of the antibiotics were performed using vitek2 (Table 1). All the isolates were 100% resistant to cephalothin MIC (>16µg/ml) and cefoxitin MIC (>16µg/ml) and 100% sensitive to ciprofloxacin MIC (>2µg/ml). 80% of isolates were resistant to ertapenem MIC (>4µg/ml) and ceftriaxone MIC (>32µg/ml) while 90% of isolates were sensitive to amikacin MIC (>32µg/ml) and 50% of bacterial isolates were sensitive to tobramycin MIC (≤4µg/ml).

From the results of antibiotics susceptibility testing, *Sphingomonas paucimobilis* was the most virulent isolate it showed 91% resistance to tested antibiotics while *Proteus mirabilis* showed a high susceptibility to tested antibiotics it was resistant to only 18% of these antibiotics.

Solvent extraction of the medicinal plants

Different solvent extracts: acetone, methanol, petroleum ether, chloroform, ethyl acetate and aqueous extract of the two medicinal plants exhibited varying degrees of inhibitory effect against 2 different Gram negative bacterial samples, as shown in Table (2). The methanolic extract of *Nigella Sativaseeds* and *Saussurea lappa* roots demonstrated the highest activity, with *Sphingomonas paucimobilis*, it showed zone of inhibition 8, 20 mm, respectively, while this extract gave zones of inhibition with *Serratia marcescens* 14, 23 mm, respectively, whereas chloroform extracts of the tested plant showed the lowest activity. Methanolic extracts were selected for further studies.

Antibacterial activity of *Nigella Sativa* seeds and *Saussurea lappa* roots extract:

Saussurea lappa roots extract was found to have more antibacterial activity than *Nigella sativa* seeds extract (Table 3). It showed antibacterial activity against *Sphingomonas paucimobilis* and *Proteus mirabilis* (*Pr.m.1*) in very low concentration 1.25 mg/ml which reveal inhibition zone of 4, 11 mm respectively. These results agree with results of Irshad *et al.*, (2011) and Thara and Zuhra, (2012). Whereas *Nigella sativa* seeds extract did not show any activity against these two isolates, these data agree with data reported by Mammad *et al.* (2017).

Extracts of both plants showed antibacterial activity against one isolate of *Ps. aureogenosa* in very low concentrations, and against *Serratia marcescens* isolates with slightly higher concentration 5mg/ml. Out of the 5 isolates of *Pseudomonas aeruginosa* only one isolate was sensitive to both extracts in as low concentration as 1.25 mg/ml. The rest of the isolates were resistant to *Nigella sativa* seeds extract and only two isolates were sensitive to high

concentrations of *Saussurea lappa* roots 10, 20 mg/ml, respectively.

The bacterial byproducts, presence of effective permeability barrier in the outer bacterial membrane, molecular genetic content, origin of isolation, and difference in bacterial type are considered the most important factors in the variation of bacterial response to the studied extract (Hasan *et al.*, 2013)

The results of antibacterial activity of tested plant extracts agree with Wang and Quinn (2010) who reported that the outer membrane of Gram negative isolates which are composed of lipopolysaccharides and phospholipids resulted in increasing the negative charge of the bacterial cell membrane and help to stabilize the structure of the membrane against antibacterial agents.

Phytochemical constituents of *Nigella sativaseeds* and *Saussurea lappa* roots extracts:

Phytochemical analysis of the methanolic extract of *Nigella sativaseeds* showed that it had high amounts of flavonoids, tannins and sterols (Table 4). The presence of these compounds may give *Nigella sativaseeds* the potentiality to act as an antibacterial agent, these results are compatible with Kamal and Ahmad (2014). The extract contained low amount of alkaloids and phenols and saponins, these were also recorded by Islam *et al.* (2012).

Methanolic extract of *Saussurea lappa* roots showed high amount of tannins in comparison with flavonoids and phenols that correlate with Prabakaran *et al.* (2011) and Zahara *et al.* (2014). However, in this study there was no appearance of alkaloids or sterols that conflict with the results reported by Pandey *et al.* (2007). Hashem and El-Kiey (1982) reported that, bacterial protein makes complex structures with

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tannins by the formation of hydrogen or covalent bond that led to inactivate microbial enzymes and cell envelope transport proteins which give tannins antibacterial power.

Conclusion:

Based on the results obtained in this study, methanolic extract of *Saussurea lappa* roots was very effective as an antibacterial agent against some MDR Gram negative bacterial isolates (*Pseudomonas aeruginosa*,

Serratia marcescens and *Proteus mirabilis* and *Sphingomonas paucimobilis*) it gave better results than the methanolic extract of *Nigella sativa* seeds. According to the significant results that obtained *in-vitro* scale, should direct us toward next step in using the extract to discover bioactive natural products as basic source for the development of modern antibiotics to overcome the problem of resistance to known traditional antimicrobial compounds and used *in-vivo* scale.

Table (1). Susceptibility to different types of antibiotics of some Gram negative bacterial isolates.

Test Organism		<i>Sphingomonas paucimobilis</i>	<i>Serratia marcescens</i>		<i>Proteus mirabilis</i>		<i>Pseudomonas aeruginosa</i>				
Antibiotic	MIC (µg/ml)	<i>Sp.p1</i>	<i>Se.m.1</i>	<i>Se.m.2</i>	<i>Pr.m. 1</i>	<i>Pr.m. 2</i>	<i>Ps.a.1</i>	<i>Ps.a.2</i>	<i>Ps.a.3</i>	<i>Ps.a.4</i>	<i>Ps.a.5</i>
Amikacin	>32	R	S	S	S	S	S	S	S	S	S
Tobramycin	≤4	R	S	R	R	S	R	R	S	S	S
Ertapenem	>4	R	R	R	S	S	R	R	R	R	R
Imipenem	>8	R	S	S	S	S	S	S	R	R	R
Meropenem	>8	R	R	S	S	S	S	S	R	R	S
Cephalothin	>16	R	R	R	R	R	R	R	R	R	R
Cefuroxime	>16	R	R	R	S	S	R	R	S	S	R
Cefoxitin	>16	R	R	R	R	R	R	R	R	R	R
Ceftazidime	>16	R	R	R	S	S	S	S	R	R	S
Ceftriaxone	>32	R	R	R	S	S	R	R	R	R	R
Cefepime	8	R	R	R	S	S	S	S	R	R	S
Ciprofloxacin	>2	S	S	S	S	S	S	S	S	S	S
% of resistance		91	67	67	25	16	50	50	67	67	50

R : resistant, S : sensitive

Sphingomonas paucimobilis (*Sp.p*), *Serratia marcescens* (*Se.m.*), *Proteus mirabilis* (*Pr.m.*), *Pseudomonas aeruginosa* (*Ps.a*)

Table (2): Antibacterial activity of plant extracts with different polar solvents

Test Organism:		<i>Sphingomonas paucimobilis</i>	
Type of plant		Diameter of inhibition zone (mm)	
Solvent		<i>Nigella sativa</i>	<i>Saussurea lappa</i>
Acetone		0	0
Ethyl acetate		5	18
Methanol		8	20
Chloroform		3	8
Petroleum ether		0	0
Negative control (Distilled water)		0	0
Test Organism:		<i>Serratia marcescens</i>	
Type of plant		Diameter of inhibition zone (mm)	
Solvent		<i>Nigella sativa</i>	<i>Saussurea lappa</i>
Acetone		0	0
Ethyl acetate		9	5
Methanol		23	14
Chloroform		12	10
Petroleum ether		0	0
Negative control (Distilled water)		0	0

Table (4): Antibacterial activity of *Nigella sativa* seeds and *Saussurea lappa* roots) extracts on the selected multi drug resistant (MDR) Gram negative isolates.

Test Organism:	<i>Sphingomonas paucimobilis</i>	<i>Proteus mirabilis</i>		<i>Serratia marcescens</i>		<i>Pseudomonas aeruginosa</i>				
Cases NO.	<i>Sp.p.1</i>	<i>Pr.m.1</i>	<i>Pr.m.2</i>	<i>Se.m.1</i>	<i>Se.m.2</i>	<i>Ps.a 1</i>	<i>Ps.a 2</i>	<i>Ps.a 3</i>	<i>Ps.a 4</i>	<i>Ps.a 5</i>
<i>Nigella sativa</i> seeds										
Extract Concentration (mg/ml)	Diameter of inhibition zone (mm)									
1.25	0	0	0	0	0	4 ±1.3	0	0	0	0
2.5	0	0	0	0	0	8 ±1.3	0	0	0	0
5.0	0	0	0	0	6 ±1.3	11 ±0.3	0	0	0	0
10.0	0	0	0	9 ±0.3	8 ±0.5	15 ±0.3	0	0	0	0
20.0	0	0	0	17 ±0.9	4 ±0.5	17 ±0.3	0	0	0	0
Positive control (Ciprofloxacin)	20 ±0.3	28 ±0.6	19 ±0.6	21 ±0.7	12 ±0.3	18 ±0.6	18 ±0.3	18 ±0.7	18 ±0.3	20 ±0.5
Negative control (S. D. water)	0	0	0	0	0	0	0	0	0	0
<i>Saussurea lappa</i> roots										
1.25	4 ±0.5	11 ±0.5	0	0	0	6 ±0.3	0	0	0	0
2.5	7 ±0.5	22 ±0.5	0	0	0	20 ±0.3	0	0	0	0
5.0	18 ±0.3	20 ±0.6	0	4 ±0.3	0	22 ±0.4	0	0	0	0
10.0	19 ±0.6	25 ±0.3	0	12 ±0.5	8 ±0.7	24 ±0.5	0	0	4 ±1.5	0
20.0	17 ±0.6	27 ±0.6	0	15 ±0.3	10 ±0.5	30 ±0.3	0	0	4 ±0.3	10 ±0.5
Positive control (Ciprofloxacin)	21 ±0.3	30 ±0.2	21 ±0.3	21 ±0.5	14 ±0.3	19 ±0.3	19 ±0.6	20 ±0.8	19 ±0.3	20 ±0.7
Negative control (S. D. water)	0	0	0	0	0	0	0	0	0	0

Sphingomonas paucimobilis (*Sp.p*), *Proteus mirabilis* (*Pr.m.*), *Serratia marcescens* (*Se.m.*), *Pseudomonas aeruginosa* (*Ps.a*)

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Table 4: Phytochemical constituents of *Nigella sativa* seeds and *Saussurea lappa* roots extracts.

Constituents	Alkaloids	Saponins	Phenols	Flavonoids	Tannins	Sterols
<i>N. sativa</i>	+	-	++	+++	+++	+++
<i>S. lappa</i>	-	-	+	+	++	-

Presence of constituent = +; Absence of constituent = -

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تأثير مستخلص الميثانول لنباتي *Nigella Sativa* و *Saussurea lappa* كمضاد للبكتريا سالبة الجرام المقاومة للعديد من المضادات الحيوية

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4 - قسم علوم الادوية كلية الطب جامعة الاسكندرية

المستخلص

تعد مقاومة البكتريا سالبة الجرام للمضادات الحيوية إحدى أهم المشاكل الصحية والإقتصادية على مستوى العالم ، مما دفع الباحثين الى البحث عن مضادات حيوية جديدة للتغلب على السلالات البكتيرية المقاومة للمضادات الحيوية والتي تزيد من معدل الوفيات والوبائية، وقد لوحظ أن الإصابة المكتسبة بالمستشفيات تتطور لدى أكثر من مليوني إنسان سنويا حيث تؤدي الى طول مدة مكوث المصاب بالمستشفى وزيادة خطورة الإصابة وازدياد التكاليف الاقتصادية.

وقد اعتمدت هذه الدراسة على استخدام مستخلصات نباتية طبيعية لها تأثير على البكتريا سالبة الجرام والمقاومة للمضادات الحيوية حيث تم تحضير أنواع مختلفة من المستخلصات لنبات *Nigella sativa* و جذور نبات *Saussurea lappa* باستخدام أنواع مختلفة من المذيبات العضوية. دلت نتائج الكشف الكيميائي إحتواء هذه المستخلصات على معظم المركبات الفعالة من القلويدات والتانينات والكلوكوسيدات والراتنجات والصابونيات والكومارينات والفلافونات .

وقد تم دراسة تأثير هذه المستخلصات على البكتريا سالبة الجرام والمقاومة للعديد من المضادات الحيوية والتي قد تم عزلها من وحدة الرعاية المركزة للأطفال حديثي الولادة من مستشفى الملك خالد بمدينة تبوك بالمملكة العربية السعودية.

تم إختبار عدد 10 عزلات بكتيرية في هذه الدراسة (5 عزلات *Pseudomonas aeruginosa* وعزلتان من كلا من *Serratia marcescens* ، *Proteus mirabilis* وعزلة واحدة من *Sphingomonas paucimobilis*) جميعهم لهم مقاومة عالية للعديد من المضادات الحيوية. وقد وجد ان المستخلص الميثانولي لجذور نبات *Saussurea lappa* هو أقوى مستخلص له تأثير على البكتريا موضع الدراسة وبشكل أكبر على *Sphingomonas sp* ، *Serratia sp* بينما وجد ان المستخلص من كلا النباتين لهما تأثير كبير ومتباين على كل من *Proteus sp* ، *Pseudomonas sp*.